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ORIGINAL ARTICLE

Molecular cloning and functional analysis of the second gene encoding glutamate dehydrogenase in triticale

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Abstract Glutamate dehydrogenase (GDH; EC 1.4.1.2) catalyzes the reductive amination of 2-oxoglutarate with ammonium and the oxidative deamination of glutamate. In some plants species, GDH is a hexamer and can be separated into seven isoenzymes that are composed of two distinct subunits: α and β. The large number of isoenzymes is the reason why GDH functions are still being intensively researched and widely studied. Until recently, the β subunit of GDH in triticale, a common Polish cereal, was thought to be encoded by the TsGDH1 gene, which can undergo posttranslational modifications and form a heterohexameric enzyme. Here, we report the cloning and molecular characterization of a second glutamate dehydrogenase gene—*TsGDH2* (encoding α subunits). The *TsGDH2* cDNA contains a 1236-bp open reading frame encoding a 411-amino-acid polypeptide with a calculated molecular mass of 44.5 kDa. To clarify the role of *TsGDH2* in triticale, we used triticale GDH α-subunit cDNA to generate transgenic *A. thaliana* lines with increased and decreased GDH activity via alternation of α-subunit levels.

Keywords Glutamate dehydrogenase · Nitrogen metabolism · Triticale · Transgenic plants

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Introduction

Glutamate dehydrogenase (GDH; EC 1.4.1.2) catalyzes reversible amination/deamination reactions in vitro, which can lead to either the synthesis or catabolism of glutamate. Because ammonium ions are one of the substrates of the amination reaction, GDH may be involved in the assimilation of ammonia. Therefore, GDH was previously considered the main ammonia (NH_4^+)-assimilating enzyme in higher plants (Bullen 1956). However, since the discovery of glutamine synthetase and glutamate synthase (GS; EC 6.1.1.3 and NADH-GOGAT; EC 1.4.1.14; Fd-GOGAT; 1.4.7.1), the GS/GOGAT cycle has been recognized as the main route of ammonium assimilation/reassimilation (Lea and Mifflin 1974). Thereafter, the physiological functions of GDH have been the subject of extensive study. Many authors believe that NADH-GDH can act in vivo as an ammonium-assimilating enzyme only under certain conditions, for instance, in dark-stressed plants, under abiotic stress, and during the processes of natural plant aging and of seed formation (Grabowska et al. 2012; Kwinta and Kolik 2006; Skopelitis et al. 2006). Other authors have suggested that GDH plays a major role in amino acid catabolism during seed germination, plant senescence and nutrient remobilization (Aubert et al. 2001; Grabowska et al. 2011; Lehmann and Ratajczak 2008).

The functions of GDH are carried out by multiple isoforms of the enzyme, although this diversity is not always possible to explain. In higher plants, GDH functions as a homohexamer or heterohexamer consisting of two subunits, α and β, that differ minimally in terms of their mass and charge. The α and β polypeptides combine in different ratios to form seven possible NADH-GDH isoenzymes in *A. thaliana*, *N. plumbaginifolia* or *V. vinifera* (Fontaine et al. 2006; Loulakakis and Roubelakis-Angelakis 1991;

Masclaux-Daubresse et al. 2002). Only one isoform was found to be present in triticale, whereas *L. luteus* contains fourteen isoforms of this enzyme (Kwinta et al. 2001; Ratajczak et al. 1986). The quantity and type of GDH isoenzymes in plants depends on the type of tissue, developmental stage and environmental growth conditions. NADH-dependent enzyme activity is usually higher in roots, developing seeds and ripening fruits compared with that in leaves (Singh and Srivastava 1982; Nanda et al. 1991; Kwinta et al. 2001). In *A. thaliana*, the most anodal isoenzyme, i.e., the homohexamer of α subunits, is active in both roots and floral stems, whereas the most cathodal isoenzyme, i.e., the homohexamer of β subunits, is active in leaves (Fontaine et al. 2006, 2013).

In most plant species, GDH is encoded by two separate nuclear genes: GDH1, also referred to as GDHB, encoding the β subunit, and GDH2, also called GDHA, encoding the α subunit (Melo-Oliveria et al. 1996; Restivo 2004; Turano et al. 1997). In some plant species, the α and β subunits may be encoded by more than one gene. For example, three genes encoding the α subunit were cloned in tomato plants (Ferraro et al. 2012), two genes encode the β subunit in rice (Qiu et al. 2009), and three genes encode the β subunit in *L. luteus* (Lehmann et al. 2011). Recent studies have shown that *A. thaliana* contains a third gene (GDH3) that encodes a γ subunit, which is expressed only in roots (Fontaine et al. 2012). The α, β and γ subunits can combine to form heterohexamers composed of the three subunits in different ratios (Fontaine et al. 2013). Such a large variety of isoenzymes provides opportunities for detailed analyses and in-depth examination of the physiological functions of individual GDH subunits. These studies are very often conducted on genetic mutants and transgenic plants that either contain additional GDH encoding genes or have these genes silenced in heterologous or homologous systems (Labboun et al. 2009; Purnell et al. 2005; Skopelitis et al. 2007; Tercé-Laforgue et al. 2013, 2015). One of the strategies to identify gene function is to perform comparative analyses of phenotypes obtained after the introduction of a gene construct that causes either gene silencing or overexpression. Purnell and Botella (2007) obtained transgenic tobacco plants (*N. tabacum*) overexpressing a gene derived from tomato (*S. lycopersicum*) encoding the β subunit. Analyses of the transformants demonstrated that GDH isoenzyme 1 (β-subunit homohexamer) exclusively catalyzes glutamic acid deamination. In contrast, Skopelitis et al. (2007) obtained tobacco plants with a gene construct derived from a vine plant (*V. vinifera*) encoding the α subunit. In this case, high deamination and amination activity was observed in plants heterologously overexpressing this gene (Skopelitis et al. 2007). Fontaine et al. (2006) applied an antisense strategy and obtained transgenic tobacco plants with impaired production of either the α- or β-subunit. A similar investigation was performed in *A. thaliana* mutants gdh1 (SALK_042736) and gdh2 (SALK_102711), which fails to express the β- and α-subunit, respectively.

The gene encoding the GDH β subunit was previously cloned in triticale (Grabowska et al. 2011). The protein product of this gene was hypothesized to undergo post-translational modifications and form a hexameric enzyme with the same electrophoretic mobility as that of the heterohexameric GDH enzyme in *A. thaliana.* In the present study, a full-length cDNA encoding GDH α subunit was isolated from triticale and designated as *TsGDH2*. Since only one GDH isoform is present in triticale, this study investigates the function of the cloned gene. To analyze the function of *TsGDH2*, transgenic *A. thaliana* plants were obtained by introducing the coding region of TsGDH2 linked to the 35S CaMV promoter, in either the sense or antisense orientation, via *Agrobacterium tumefaciens* transformation. We generated transgenic *A. thaliana* lines with increased and decreased GDH activity via alternation of α-subunit levels.

Materials and methods

Plant material and growth conditions

Seeds of triticale (×*Triticosecale* Wittm.) cv. Witon were obtained from the Breeding Station in Laski, Poland. The grains were surface sterilized with 10% hypochlorite and then germinated in darkness at 20 °C and 100% relative humidity. Samples were collected after 72 h of germination, immediately frozen in liquid nitrogen and stored at −80 °C until use.

The seeds of the *A. thaliana* ecotype Columbia (Col-0) were obtained from the Nottingham Arabidopsis Stock Center. Germination of the seeds was synchronized with treatment at 4 °C for 48 h in the dark. Plants were grown to the flowering stage in a growth chamber (Versatile Environmental Test Chamber MLR-35OH, Sanyo, UK) at 22 °C with 10 h light (250 μE m^{-2} s^{-1}) during the vegetative stage and 14 h light during generative growth.

Cloning procedures

The RNA was isolated from the shoots and roots of 3-day-old triticale seedlings using the guanidinium thiocyanate/acidic phenol-extraction method (Chomczynski and Saschi 2006). To eliminate any genomic DNA contamination, each RNA sample was treated with RNAse-free DNAse I (Fermentas). First-strand cDNA was synthesized using 2 μg of total RNA primed with an oligo$(dT)_{12–18}$ primer and an avian myeloblastosis virus reverse transcriptase

(AMV RT) following the manufacturer's protocol (Promega).

Two oligonucleotide primers, GDH2-F1 (5′GGCTCTC CTGGCCGAATATGGGAAGTCT3′) and GDH2-R1 (5′CCTAAGGTTGCAATCTTGAGACTT3′), were used to amplify the internal region of *TsGDH2* cDNA with GoTaq Flexi DNA Polymerase (Promega). Both primers corresponded to a fragment of the *Brachypodium distachyon* gene encoding glutamate dehydrogenase 2 (GenBank accession number: XM_003580211). PCR reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research) under the following conditions: 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C, and a final extension step for 7 min. The PCR amplification product was separated by agarose gel electrophoresis and extracted with a Silica Bead DNA gel Extraction kit (Thermo Scientific). The resulting amplified fragment was cloned into pGEM-T Easy (Promega) and sequenced.

Full-length *TsGDH2* cDNA was obtained using the GeneRacer Kit (Invitrogen). The gene specific primers used for RACE were designed from the above partial *TsGDH2* cDNA sequence. The primer GDH2-R2 (5′AATAACAAAGGTTGATCCAGAAATAGAC3′) was used for 5′ RACE, and GDH2-F2 (5′-GTACCTGCTCTGATGAAGCACAGAAAT3′) was used for 3′ RACE. PCR reactions were performed under the following conditions: 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min at 68 °C, and a final 7 min at 68 °C. The amplification was performed with Platinum *Taq* DNA Polymerase (Invitrogen). The resulting amplified fragment was cloned using the TOPO TA Cloning Kit (Invitrogen) and sequenced. Sequencing was performed with the ABI Prism BigDye Terminator Cycle Sequencing Kit on the ABI Prism 3730 DNA analyzer (Applied Biosystems), at the DNA Sequencing and Oligonucleotide Synthesis Laboratory, The Institute of Biochemistry and Biophysics, Polish Academy of Sciences.

Bioinformatics analysis

The obtained nucleotide sequences date from this article have been deposited in GenBank. Sequences were verified by a database search in the National Center for Biotechnology Information server using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). The deduction of the amino acid sequence, calculation of the theoretical molecular mass and pI was performed with ExPASy (http://www.expasy.ch/tools/). Multiple-sequence alignments of GDH amino acids sequences were generated using the CLUSTAL W program (Thompson et al. 1997). A phylogenetic analysis was performed using the neighbor-joining (NJ) method, as implemented in the PhyML program (http://www.phylogeny.fr/version2_cgi/index.cgi) (Dereeper et al. 2008).

Gene constructs

Agrobacterium tumefaciens strain EHA105 carrying pCAMBIA1380 was used to transform *A. thaliana* plants. The TsGDH2 coding sequence (1236 bp) was amplified with gene specific primers (forward: 5′-ggaaagctt/ggaaagctt-ATGAACGCGCTCGCCGCGACCAGCCGC3′; reverse: 5′taggttacc/ggcgaattc-TCATGCCTCCCAGCCC CTCAAGAT3′). *Hin*dIII and *Eco*91I or *Hin*dIII and *Eco*RI restriction sites were introduced at the ends of the primers for the sense and antisense orientations of *TsGDH2*, respectively. The amplification was performed with GoTaq Flexi DNA Polymerase (Promega). PCR reactions were performed under the following conditions: 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 68 °C, 60 s at 72 °C, and a final 5 min at 72 °C. After a restriction analysis, purified fragments were ligated to the pCAMBIA1380 vector. These T-DNA constructs were designated as *35S::TsGDH2s* for the sense and *35S::TsGDH2as* for the antisense orientation. All constructs for transformation were verified by sequencing.

Plant transformation

The floral dip method was used for plant transformation (Clough and Bent 1998). Plants designated as T_0 were grown to maturity, and their seeds were harvested. Seeds from transformed plants (T_1 generation) were surface sterilized by immersion in 96% (v/v) ethanol for 1 min, washed with sterile water and then diluted in bleach solution (final concentration 20% v/v sodium hypochlorite) for 5 min, washed five times with sterile water, suspended in 0.1% sterile agarose and plated on selective medium (1/2 MS) (Murashige and Skoog 1962) containing 1% sucrose, 0.8% agar, 20 mg L^{-1} hygromycin, and 200 mg L^{-1} cefotaxime. The growth conditions were as described above. After 2 weeks, resistant seedlings were transferred to fresh selection medium with 1.5% agar. After 1 week, seedlings were transferred to soil. The T_2 seeds from each T_1 plant were harvested. Progeny (T_3) were selected from each T_2 line that showed a 3:1 ratio of hygromycin resistance consistent with single-locus insertion of the transgene.

Analysis of *A. thaliana* transformants

Genomic DNA was isolated from T_3 transformants and wild-type plants (control) using the CTAB extraction protocol adapted from Weigel and Glazebrook (2009). Antibiotic-resistant plants were screened by PCR for the hygromycin phosphotransferase gene (*hpt*) (primers: 5′GGCGAGTACTTCTACACA3′ and 5′GCGAAG AATCTCGTGCTT3′), the 35S promoter (primers:

5′CATGGAGTCAAAGATTCAAATAGAGGA3′ and 5′CTCTCCAAATGAAATGAACTTCCTTA3′) and the *TsGDH2* cDNA sequence (primers: 5′ATGAACGCGCTCGCCGCGACCAGCCGC3′ and 5′TCATGCCTCCCAGCCCCTCAAGAT3′). PCR reactions were performed under the following conditions: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C (for *hpt* gene and 35S promoter) or 65 °C (for *TsGDH2* gene), 30 s (for *hpt* gene and 35S promoter) or 1 min (for *TsGDH2* gene) at 72 °C, and a final 3 min at 72 °C. Amplification was performed with GoTaq Flexi DNA Polymerase (Promega).

Expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from 100 mg of young leaves of *A. thaliana* transgenic lines and control plants. Semi-quantitative RT-PCR analysis was performed using the One-Step RT-PCR Kit (Novagen) according to the manufacturer's instructions. In the expression analysis, three primer pairs were used (Table 1). The first pair was designed for the triticale gene (*TsGDH2*), the second for the endogenous *GDH2* gene (AT5G07440), and the third pair for the reference *Actin 2* gene in *A. thaliana*. Reactions were performed under the following conditions: 30 min at 60 °C, 2 min at 94 °C, 35 cycles (for *TsGDH2*, *GDH2*) and 30 cycles (for *Actin 2*) of 30 s at 94 °C, 30 s at *X* °C (Table 1), 30 s at 72 °C and a final extension step for 5 min at 72 °C. Each sample was analyzed with at least three independent experiments. The amplified products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide.

Protein extraction, enzyme assays and electrophoresis

GDH was extracted by homogenizing 100 mg of leaves in 1 mL of extraction buffer containing 100 mM Tris–HCl (pH 7.6), 5 mM 2-mercaptoethanol and 20 µM PMSF. The homogenates were centrifuged for 15 min at 13,000×*g* at 4 °C, and the supernatant was used as the enzyme extract. GDH activity was determined in both the aminating (NADH-GDH) and deaminating (NAD^+-GDH) directions according to the method of Barash et al. (1973). The standard amination reaction mixture contained 100 mM Tris–HCl (pH 8.3), 200 mM NH_4Cl, 0.28 mM NADH, 32 mM 2-oxoglutarate, 0.05 mL of enzyme extract and deionized water to a final volume of 0.6 mL. The standard deamination reaction mixture contained 100 mM Tris–HCl (pH 9.2), 200 mM L-glutamate, 0.25 mM NAD^+, 0.05 mL of enzyme extract and deionized water to a final volume of 0.6 mL. All assays were performed at 30 °C. One unit of GDH activity was defined as the reduction or oxidation of 1 µmol of coenzyme (NAD^+ or NADH, respectively) min^{-1} g^{-1} DW (dry weight). Enzyme activity measurements are presented as the mean ± SD for three independent experiments, with two replicates each. Statistical significance was determined using Student's *t* test.

Native PAGE of GDH extracts was performed using the modified Laemmli method (1970) with 7.5% resolving and 4% stacking gels. Bands with GDH activity were visualized on the gel using the tetrazolium system (Lehmann et al. 1990).

Protein concentration was determined colorimetrically according to the Bradford method (1976) using bovine serum albumin as a standard.

Results

Sequence analyses of *TsGDH2*

The *TsGDH2* gene was isolated using RT-PCR. The full-length *TsGDH2* cDNA (Gene Bank, accession number: KU311056) comprised 1538 bp, with a 1236-bp open reading frame (ORF) and a 302-bp 3′untranslated region (3′UTR). The putative protein sequence of TsGDH2 contained 411 amino acids, with a calculated molecular mass of 44.5 kDa and a predicated isoelectric point (pI) of 6.20. The deduced amino acid sequence of TsGDH2 was screened for conserved domains at the Conserved Domain Database at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Two features were present in the TsGDH2 protein: an NAD(P) binding domain of glutamate dehydrogenase from Gly-176 to Arg-402 and a Glu/Leu/Phe/

Table 1 Primer sequences and PCR conditions used for the semi-quantitative RT-PCR analysis

Gene	Forward and reverse primers	Product length (bp)	*X* annealing temperature (°C)	PCR cycle number
TsGDH2	5′TCTCCCGGTGATTTAAGTAGGAGT3′ 5′AAAGGTTGATCCAGAAATAGAC3′	306	62	35
GDH2	5′GGGAAATCGATTCAGGGTTTGACA3′ 5′TGAAATCATTGAGACTTCCAGT3′	199	58	35
Actin 2	5′GGCGATGAAGCTCAATCCAAACG3′ 5′GGTCACGACCAGCAAGATCAAGACG3′	380	55	30

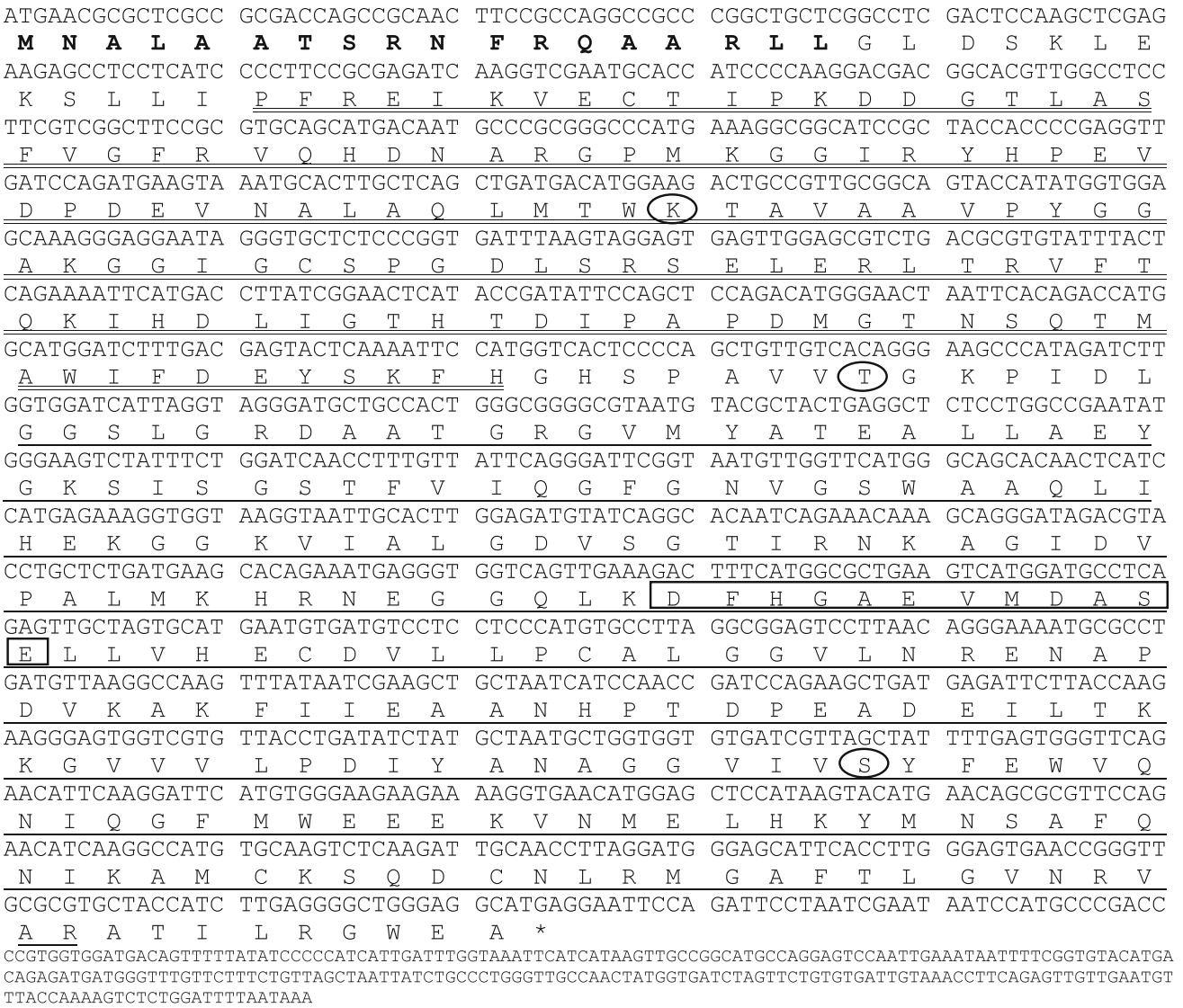

Fig. 1 The nucleotide and putative amino acid sequences of TsGDH2. The mitochondrial target peptide is in *bold*, the NAD(P)-binding domain of glutamate dehydrogenase is *underlined*, the dehydrogenase dimerization domain is *double underlined*, the three amino acids residues involved in Glu binding are marked by *an ellipse*, and the region containing the EF-hand loop motif is *boxed*

Val dehydrogenase (ELFV_dehydrog_N, pfam02812) dimerization domain from Pro-31 to His-161 (Marchler-Bauer et al. 2013). Three conserved amino acids residues, Lys-90, Thr-169 and Ser-344, are involved in Glu binding according to Britton et al. (1992). In TsGDH2, the region Asp-265 to Glu-276 resembles an EF-hand loop motif, which is associated with Ca^{2+} binding in other proteins (Denessiouk et al. 2014). Additional the TargetP program predicted that TsGDH2 contains an 18-residue mitochondrial target peptide (mTP) at the N terminus (score 0.795, reliability class 2) (Fig. 1).

The overall sequence alignment analysis revealed that TsGDH2 is highly homologous to GDHs in other plants (Supplementary Material Fig. 1). The TsGDH2 protein showed high identity to the selected GDH of *B. distachyon* (XP_003580259, 94% identity, score 2058), *O. sativa* (BAE48298.1, 93% identity, score 2044), *N. sylvestris* (XP_009758042.1, 82% identity, score 1834), *N. plumbaginifolia* (CAA69601, 79% identity, score 1777), *V. vinifera* (XP_010662019.1, 82% identity, score 1812). The deduced sequence of TsGDH2 and *A. thaliana* GDH2 (NP_196361.1) showed 83% identity and a score of 1861, whereas TsGDH2 and *A. thaliana* GDH1 (NP_197318.1) showed 79% identity and a score of 1760 (Fig. 2).

In the majority of plant species, GDH is encoded by at least two genes encoding the α and β subunits that make up the enzyme. To determine which subunit (α or β) is encoded by *TsGDH2*, as well as the evolutionary relationship between TsGDH2 and GDHs from other plants, a phylogenetic tree was constructed using the neighbor-

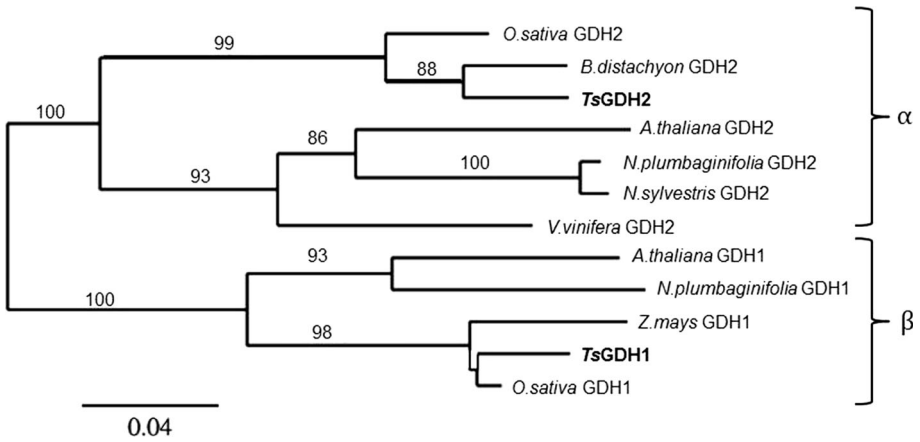

Fig. 2 Phylogenetic tree of glutamate dehydrogenase from different plant species. *Numbers above the branches* represent bootstrap support for 100 replicates. The analysis was based on the deduced amino acid sequences of GDH genes. *Oryza sativa—GDH2* (AB189166.1); *Brachypodium distachyon—GDH2* (XM_003580211); ×*Triticosecale—GDH2* (KU311056); *Arabidopsis thaliana—GDH2* (NM_120826.2); *Nicotiana plumbaginifolia—GDH2* (Y08293.2); *Nicotiana sylvestris—GDH2* (XM_009759740.1); *Vitis vinifera—GDH2* (AJ303070.1); *Arabidopsis thaliana—GDH1* (NM_121822.3); *Nicotiana plumbaginifolia—GDH1* (Y08292.1); *Zea mays—GDH1* (NM_001111831.1); ×*Triticosecale—GDH1* (HQ658905.1)

joining method. The phylogenetic tree was generated using the deduced amino acid sequences of monocot and dicot GDHs, encoding both α and β subunits. The phylogenetic analysis indicated that GDH2 (α-subunit) and GDH1 (β-subunit) were each classified into one group containing all known members. Each group contained sequences from monocots and dicots, suggesting that the genes were grouped according to the nature of the subunit encoded, rather than according to the species of origin. The phylogenetic analysis clearly indicated that TsGDH2 belongs to the GDH2-NAD(H) subgroup.

Molecular analysis of *A. thaliana* transformants

The binary vectors *35S::TsGDH2s* and 35S::*TsGDH2as* containing the cauliflower mosaic virus (CaMV) 35S promoter upstream of the *x Triticosecale TsGDH2* gene in the sense and antisense orientation, respectively, were used to transform *A. thaliana* plants. After *Agrobacterium* transformation and hygromycin selection, several transformants were produced. Four transgenic lines (A7, A15—*TsGDH2* antisense; S3, S4—*TsGDH2* sense) were grown up to the T_3 generation, from which homozygous plants (for *hpt* marker) were analyzed. All T_3 lines had a single T-DNA insertion locus that segregated in a Mendelian fashion (results not shown). No phenotypic changes were observed transgenic plants compared with controls. Stable transformation of the transgenes was confirmed by PCR. In all the transformants into which the *35S::TsGDH2s* and *35S::TsGDH2a* cassettes were introduced, 887-bp (for the *hpt* gene), 525-bp (for the 35S promoter) and 1254-bp (for *TsGDH2* gene) products were detected, indicating correct integration of the T-DNA into all the transgenic lines. DNA

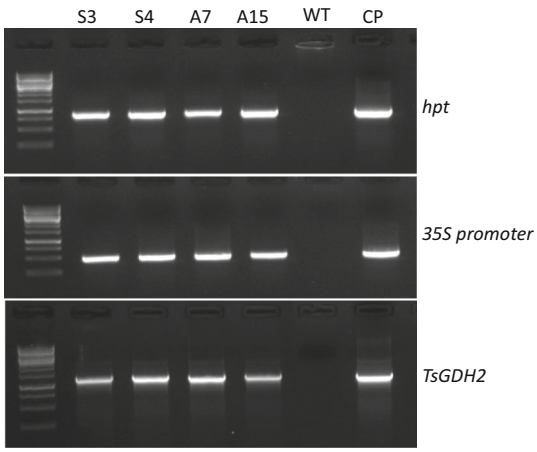

Fig. 3 PCR analysis of Arabidopsis transgenic lines. DNA was isolated from transgenic plants transformed with the 35S::TsGDH2s (S3 and S4) or the 35S::TsGDH2as (A7 and A15) cassette. PCR amplification was performed using primers for the *hpt* gene, the *35S* promoter and the *TsGDH2* gene. WT-negative control, DNA from wild-type plants; CP-positive control, plasmid DNA (pCAMBIA1380 with *TsGDH2*); DNA molecular markers (SMO311; Thermo Scientific)

isolated from non-transformed plants (WT) served as a negative control, whereas the plasmid DNA (pCAMBIA1380 with *TsGDH2*) was used as a positive control (Fig. 3).

Expression analysis by semi-quantitative RT-PCR and modulation of GDH activity in transgenic plants

To evaluate the effects of *TsGDH2* overexpression and antisense repression, the expression levels of the introduced gene and the *GDH2* (AT5G07440) gene were

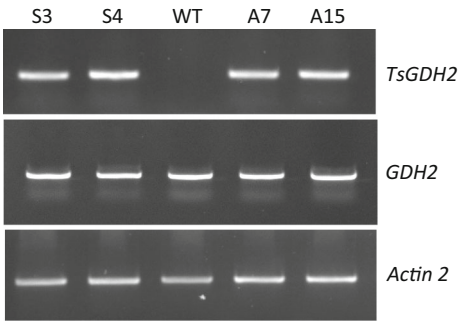

Fig. 4 Semi-quantitative RT-PCR analysis of the *TsGDH2* and *GDH2* (AT5G07440) genes. Total RNA was isolated from transgenic plants transformed with the 35S::*TsGDH2s* (S3 and S4) and the 35S::*TsGDH2as* (A7 and A15) cassette and from wild-type (WT) plants. The *Actin 2* gene (AT3G18780) was used to normalize the template amounts in different samples. Each figure is representative of three separate amplification experiments that yielded similar results

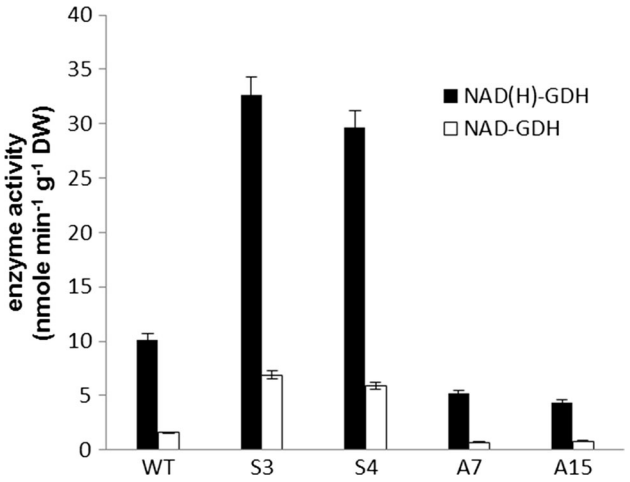

Fig. 5 GDH activity of wild-type Arabidopsis plants (WT) and transgenic lines: S3 and S4 (*35S::TsGDH2s*), A7 and A15 (*35S::TsGDH2as*). Enzyme activities were measured in leaf samples. NAD(H)-GDH aminating activity (*black columns*), NAD-GDH deaminating activity (*white columns*). GDH activity was measured on three individual plants for each line. Values are mean ± SE

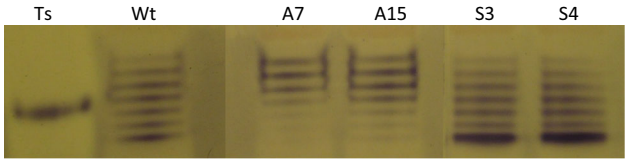

Fig. 6 NAD-GDH isoenzyme pattern of wild-type Arabidopsis plants (WT), transgenic lines [A7 and A15 (*35S::TsGDH2as*) and S3 and S4 (*35S::TsGDH2s*)] and triticale leaves (Ts). The soluble protein extracts of leaves were subjected to native PAGE followed by NAD-GDH in-gel deaminating activity staining. The amount of protein loaded in each lane was calculated on a similar dry-weight basis for each leaf sample. On the *left-hand side* of the gel, the isoenzyme pattern from triticale leaf extracts (Ts) was used as a marker to verify the corresponding isoenzymes in Arabidopsis. In-gel activity staining was performed on three individual plants for each line

analyzed. The transcription level of *TsGDH2* and *GDH2* genes was determined via semi-quantitative RT-PCR analysis. The results were standardized relative to the *Actin 2* (AT3G18780) gene from *A. thaliana*. Total RNA from the leaves of plants transformed with sense and antisense constructs, as well as from control plants, was used. As seen in Fig. 4, both sets of transformants containing either the sense or antisense constructs exhibited high levels of the corresponding *TsGDH2* transcript, whereas wild-type plants did not express the *TsGDH2* gene. With regard to endogenous *GDH2* gene expression, similar transcript levels were observed in the transgenic and the control plants. Following *TsGDH2* and *GDH2* expression analysis in the transformants, the next step was to measure both NADH-dependent aminating and NAD-dependent deaminating GDH activity (Fig. 5). In the two overexpressing transgenic lines, the aminating activity was increased 3.2-fold (S3 line) and 2.9-fold (S4 line), and the deaminating activity was increased 4.3-fold (S3 line) and 3.7-fold (S4 line) compared with the wild-type (WT) control plants. However, in the antisense lines, the aminating and deaminating activities were reduced 1.8-fold (A7 line), 2.3-fold (A15 line), 2.2-fold (A7 line) and 2.0-fold (A15 line) compared with the wild-type (WT) control plants.

In-gel activity staining was used to detect the GDH isoenzyme compositions in the leaves of transgenic plants (Fig. 6). Overexpression of the α-subunit polypeptide in *35S::TsGDH2s* transformants (S3 and S4) resulted in increased abundance of the anionic isoenzymes, especially the homohexameric isoenzyme 7. The other bands of GDH activity were stronger compared with the control plants. In the two transgenic antisense lines (A7, A15), only the most cathodal isoenzymes (GDH1, 2, 3 and 4) were visible; the other activity bands were much weaker compared with the wild-type plants. For comparative purposes, in addition to the transgenic lines and wild-type *A. thaliana*, triticale was also analyzed. Only one enzyme isoform, characterized by medium electrophoretic mobility, was observed. This isoform corresponds to isoenzyme 4 of *A. thaliana*—a heterohexamer composed of three α-subunits and three β-subunits.

Discussion

The full-length cDNA of *TsGDH2*, encoding an NAD(H)-GDH in triticale, was identified and characterized. This gene is the second GDH to be cloned in triticale. Previous studies carried out by Grabowska et al. (2011, 2012) analyzed the *TsGDH1* gene. Sequence alignments of the previously cloned full-length *TsGDH1* gene (Grabowska et al.

2011) and *TsGDH2* indicated 71% nucleotide sequence similarity and 81% similarity within the 411-amino-acid sequence (Supplementary Material Figs. 2, 3). Comparable sequence similarity is observed in *A. thaliana*, where the sequence identity between *GDH1* and *GDH2* at the nucleotide and amino acid levels is 75 and 81%, respectively (Turano et al. 1997). The deduced amino acid sequence of the *TsGDH2* contains a consensus calcium binding EF-loop motif between Asp-265 and Glu-276 (Grabarek 2006). This sequence is characteristic for the GDH α-subunit but is absent from the GDH β-subunit. The region responsible for binding calcium ions has also been identified in the genes encoding the GDH α-subunit in other species, for example, in *A. thaliana*, *N. tabacum*, and *S. lycopersicum* (Ferraro et al. 2012; Purnell et al. 2005; Turano et al. 1997). Research on subcellular localization prediction has demonstrated that GDH is mostly localized in the mitochondria of the phloem companion cells (Loulakakis and Roubelakis-Angelakis 1990; Paczek et al. 2002). Analyses of the deduced amino acid sequence of *TsGDH2* revealed the presence of a signal peptide directing the protein to mitochondria. In tomato plants, in which three genes encoding the GDH α subunit were cloned, predicted mitochondrial target peptides were also identified at the N terminus (Ferraro et al. 2012).

Phylogenetic analyses distinguishes two distinct groups of GDH genes. Genes encoding the α-subunit belonged to the first group, whereas genes encoding the β-subunit were clustered in the second group. The phylogenetic analysis of the deduced amino acid sequences encoded by the GDH2 genes identified in triticale and the sequences of α- and β-GDH subunits from selected higher plants confirmed that *TsGDH2* encode the α-type subunit.

To elucidate the function of the *TsGDH2* gene of triticale, which shares high sequence identity with other genes coding for the GDH α subunit, an antisense repression and overexpression strategy in *A. thaliana* was used. Analyses of transgenic tobacco plants with modified expression of genes encoding GDH α and β subunits revealed various metabolic functions of GDH isoenzymes and indicated that, under normal growth conditions, GDH isoenzyme 1 (β-homohexamer) only deaminates glutamate, whereas GDH isoenzyme 7 (α-homohexamer) exhibits high deaminating activity but also weak aminating activity (Purnell et al. 2005; Purnell and Botella 2007; Skopelitis et al. 2007).

In the present work, *A. thaliana* plants were transformed using T-DNA constructs containing the full-length *TsGDH2* gene coding for the triticale α-GDH subunit in the sense and antisense orientation. In all the analyzed lines, the transcript of the inserted *TsGDH2* gene was present, suggests that the T-DNA was successfully transferred into the *A. thaliana* genome and then transcribed. In the control group, no *TsGDH2* transcript was detected. Furthermore, the levels of endogenous *GDH2* gene transcript were similar in the transgenic plants and in the control group. In the overexpressing transgenic lines (S3 and S4), increased GDH aminating and deaminating activity was observed in vitro compared with the control plants. Furthermore, the increase in enzyme activity measured in vitro in lines S3 and S4 was similar to that detected by native gel staining, and the expression of *TsGDH2* was consistent with the synthesis of the GDH α-subunit, especially GDH isoenzyme 7. Skopelitis et al. (2007) obtained similar results, namely, increased GDH activity, upon inserting a *Vvgdh-NAD* derived from grapevine and encoding the α-GDH subunit into transgenic tobacco plants. The in vitro aminating activities were very high in overexpressing transgenic lines. Furthermore, overexpression of the α-subunit resulted in increased abundance of anionic isoenzymes.

In work conducted by Purnell et al. (2005), a gene encoding the β subunit in tomato plants was introduced into the antisense tobacco line. In this study, no transcript of the introduced gene was found; however, reduced GDH activity was observed. The mechanism of gene expression inhibition typically relies on the formation of duplexes between the antisense RNA fragments and the endogenous mRNA. Due to the low level of complementarity between RNA in the formed duplexes, the endogenous RNA is not silenced; therefore, the transcript was still detectable but interfered with the translation of endogenous transcripts responsible for the synthesis of the β subunit. Moreover, according to gene-silencing studies concerning carried out by Cannon et al. (1990) and Oeller et al. (1991), during inhibition by antisense RNA expression, reduced expression of endogenous gene is often observed. Therefore, a different silencing model was proposed in which antisense RNA forms duplexes with the corresponding endogenous sense RNA. Thus, the duplexes are then degraded by RNase activity, resulting in the loss of the target mRNA (Mol et al. 1990). In the obtained A3 and A4 lines, expression of the antisense gene and the endogenous gene coding for the α subunit was observed. Thus, the heteroduplexes that might have formed were likely stable. Related results were found by Temple et al. (1993), who transformed tobacco plants in a heterologous system using an antisense genetic construct containing a glutamate synthetase gene derived from the alfalfa plant. In the transgenic tobacco plants, neither the endogenous gene transcript nor the introduced alfalfa gene was observed. The level of GS polypeptides was found to be reduced. In both above studies, the formed heteroduplexes were not used in the translation process, which in our experiment resulted in decreased aminating and deaminating GDH activity in vitro compared with the control plants. These results were also confirmed by native PAGE NAD-GDH gel activity staining, in which only the most cathodal

isoenzymes (GDH 1, 2, 3 and 4) were observed, and the activity of the remaining isoenzymes was lower compared with the control. These results demonstrate that the full-length triticale TsGDH2 gene, when transcribed in the antisense orientation, is capable of downregulating GDH2 and GDH1 in *A. thaliana* leaves. Therefore, the antisense RNA approach is effective for silencing gene expression even in a heterologous system. TsGDH2 shares 83 and 79% nucleotide sequence homology with GDH2 and GDH1 from *A. thaliana*. Because the sequence similarity is so large, both genes could have been silenced to some extent, and this result was confirmed by gel electrophoresis.

In summary, a second triticale gene, encoding GDH α subunit, was cloned. To assign a function to this gene, *A. thaliana* plants were transformed with two genetic constructs in the sense and antisense orientation. The resulting transgenic *A. thaliana* plants were characterized by an altered level of GDH-NAD(H) activity due to the alteration of α-subunit levels, which indicates that the TsGDH2 protein is functionally analogous to GDH2 in other plants. Our future studies will focus on two areas. First, the transgenic lines will be analyzed further using TsGDH2-specific antibodies. The second aim will be to obtain transgenic *A. thaliana* plants using the β subunit-coding gene (*TsGDH1*) in triticale to further clarify the in vivo reaction direction(s), physiological role(s) and regulation of the GDH isoenzymes in triticale. This work is of particular interest, as triticale only contains one enzyme isoform that might be composed of three α and three β subunits.

Author contribution statement AG conceived and designed the experiments; AG performed the experiments with help JK and EZZ; AG and JK analyzed the data; EK technical support; JK revised the manuscript, AG wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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